Journal of Food Protection, Vol. 68, No. 5, 2005, Pages 906–912

Biofilm Formation, Cellulose Production, and Curli Biosynthesis by *Salmonella* Originating from Produce, Animal, and Clinical Sources†

ETHAN B. SOLOMON, BRENDAN A. NIEMIRA, GERALD M. SAPERS, AND BASSAM A. ANNOUS*

Food Safety Intervention Technologies Research Unit, U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, Pennsylvania 19038-8598, USA

MS 04-477: Received 12 October 2004/Accepted 10 January 2005

ABSTRACT

The ability of 71 strains of *Salmonella enterica* originating from produce, meat, or clinical sources to form biofilms was investigated. A crystal violet binding assay demonstrated no significant differences in biofilm formation by isolates from any source when tested in any of the following three media: Luria-Bertani broth supplemented with 2% glucose, tryptic soy broth (TSB), or 1/20th-strength TSB. Incubation was overnight at 30°C under static conditions. Curli production and cellulose production were monitored by assessing morphotypes on Luria-Bertani agar without salt containing Congo red and by assessing fluorescence on Luria-Bertani agar containing calcofluor, respectively. One hundred percent of the clinical isolates exhibited curli biosynthesis, and 73% demonstrated cellulose production. All meat-related isolates formed curli, and 84% produced cellulose. A total of 80% of produce-related isolates produced curli, but only 52% produced cellulose. Crystal violet binding was not statistically different between isolates representing the three morphotypes when grown in TSB; however, significant differences were observed when strains were cultured in the two other media tested. These data demonstrate that the ability to form biofilms is not dependent on the source of the test isolate and suggest a relationship between crystal violet binding and morphotype, with curli- and cellulose-deficient isolates being least effective in biofilm formation.

Foodborne salmonellosis is responsible for an estimated 1.3 million illnesses and 553 deaths per year in the United States *(16)*. While meat, poultry, and eggs remain the leading vehicles for outbreaks, *Salmonella* has been isolated with increasing frequency from fresh produce in recent years *(3)*. A number of studies have demonstrated the ability of *Salmonella* to attach and form biofilms on a wide variety of food contact surfaces, including metal, plastic, and rubber *(14, 26, 31)*. Sanitizers tested in our laboratory have proven incapable of inactivating *Salmonella* inoculated onto cantaloupe, especially when the organism was allowed to reside on the melon surface for more than 48 h *(33)*. In addition, the attachment strength of *Salmonella* on cantaloupe surfaces increased with increased contact time *(32)*. These results led to the hypothesis that salmonellae inoculated onto melon surfaces rapidly form biofilms. Scanning electron microscopy demonstrated biofilm formation by *Salmonella* Poona RM 2350 on cantaloupe rind tissue after just 24 h of storage *(1)*. The entrapment of cells of *Salmonella* within a biofilm is likely responsible for enhanced sanitizer resistance and increased attachment strength.

A biofilm is generally defined as ''a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface'' *(6)*. The presence of native microbial biofilms has been visualized on a wide variety of plant surfaces, including endive, parsley, spinach, basil, leeks, and a variety of sprouts *(10, 11, 20, 21)*. The formation of biofilms by bacteria on plants likely improves the ability of these organisms to colonize and survive the harsh environment of the phyllosphere *(19)*.

Biofilm formation by *Salmonella* has only recently been investigated. Early work documented the production of thin aggregative fimbriae (curli) by virulent strains *(25)*, but a conclusive role for these fibers was not elucidated. The production of curli was later found to be an important component in the formation of an extracellular matrix by cells of *Salmonella (24)*. Recently, a second component of this matrix was found to be cellulose *(22, 34)*. The production of cellulose and curli by *Salmonella* leads to a matrix of tightly packed cells covered in a hydrophobic network. This network of material is important in biofilm formation as well as in its persistence on various surfaces *(5, 27)*. While in vivo studies have shown that the production of cellulose may not be involved in the virulence of *Salmonella* Enteritidis, cellulose-deficient mutants were less persistent and more susceptible to chlorine treatments in solution *(27)*. These results indicate that cellulose and curli may play a role in the survival and resistance of salmonellae in the food environment.

The recent increase in produce-related outbreaks of salmonellosis, along with the indication that *Salmonella* readily forms biofilms on cantaloupe surfaces *(1)*, gives rise to the hypothesis that perhaps produce-related isolates are able to survive in the harsh phyllosphere environment by exhib-

* Author for correspondence. Tel: 215-233-6797; Fax: 215-233-6406; E-mail: bannous@errc.ars.usda.gov.

† Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

TABLE 1. *Biofilm formation by clinical isolates of* Salmonella

Salmonella serotypes	Biofilm formation[a]			Morphotype on Congo red[b]	Fluorescence on LB + calcofluor[c]
	LB + 2% glucose	TSB	1/20-TSB		
Branderup H0663	0.153 ± 0.045	0.568 ± 0.039	0.473 ± 0.024	rdar	+
Enteritidis H3502	0.015 ± 0.003	0.510 ± 0.027	0.680 ± 0.034	rdar	+
Enteritidis H3526	0.038 ± 0.005	0.370 ± 0.059	0.382 ± 0.037	bdar	−
Enteritidis H3527	0.08 ± 0.009	0.181 ± 0.029	0.281 ± 0.038	rdar	+
Enteritidis H4386	0.092 ± 0.022	0.401 ± 0.062	0.423 ± 0.041	rdar	+
Hadar 110-96	0.161 ± 0.013	0.361 ± 0.026	0.602 ± 0.027	bdar	−
Newport H1073	0.07 ± 0.008	0.589 ± 0.026	0.372 ± 0.016	bdar	−
Thompson H2464	0.09 ± 0.012	0.625 ± 0.028	0.702 ± 0.028	rdar	+
Typhimurium H3379	0.091 ± 0.016	0.309 ± 0.037	0.177 ± 0.008	bdar	−
Typhimurium G7601	0.031 ± 0.005	0.229 ± 0.028	0.659 ± 0.067	rdar	+
Typhimurium G8430	0.019 ± 0.003	0.407 ± 0.062	0.491 ± 0.049	rdar	+
Typhimurium H2662	0.009 ± 0.002	0.385 ± 0.053	0.571 ± 0.056	rdar	+
Typhimurium H3278	0.046 ± 0.006	0.250 ± 0.035	0.727 ± 0.077	rdar	+
Typhimurium H3380	0.027 ± 0.003	0.154 ± 0.023	0.527 ± 0.069	rdar	+
Typhimurium H3402	0.038 ± 0.007	0.262 ± 0.032	0.965 ± 0.100	rdar	+

[a] Average OD (590 nm) ± standard error from two separate experiments.
[b] rdar, red, dry, and rough morphotype indicating curli and cellulose production; bdar, brown, dry, and rough morphotype indicating curli production but lack of cellulose synthesis.
[c] Under long-wave UV (366 nm).

iting enhanced biofilm formation. Furthermore, curli and cellulose production by produce-related *Salmonella* has not been investigated. Therefore, the objectives of our study were to (i) determine whether produce-related strains exhibited enhanced biofilm formation in vitro compared to meat-related and clinical isolates, and (ii) screen our collection for cellulose and curli production.

MATERIALS AND METHODS

Bacteria. *Salmonella enterica* serovar Enteritidis 3934, 942, 1170/97, and 1162/97 were obtained from Dr. Carlos Gamazo (Department of Microbiology, University of Navarra, Spain). These isolates were used as comparison strains, as their cellulose and curli production has already been documented *(28).* All other isolates were from the U.S. Department of Agriculture–Agricultural Research Service–Eastern Regional Research Center culture collection. A total of 15 clinical isolates (Table 1), 25 produce isolates (Table 2), and 31 meat isolates (Table 3) were used in this study. Stocks were stored in tryptic soy broth (TSB; BBL/Becton Dickinson, Sparks, Md.) containing 30% glycerol at −80°C. Working cultures were maintained on tryptic soy agar (BBL/Becton Dickinson) slants at 4°C.

Quantification of biofilm formation. Biofilm formation was quantitated in Luria-Bertani (LB) broth supplemented with 2% glucose, TSB, and 1/20th-strength TSB (1/20-TSB) as previously described *(17),* with the addition of a fixation step (80°C for 30 min) prior to staining with crystal violet. Heat fixation has been used in crystal violet assays to measure biofilm formation, and we found that it provided more reproducible results *(12).* Briefly, cells were cultured overnight in the appropriate medium and then diluted (1:10) and inoculated into 96-well polystyrene microtiter plates (Becton Dickinson, Franklin Lakes, N.J.). Plates were incubated overnight at 30°C under static conditions, and biofilm formation was quantitated. Crystal violet–stained biofilms were solubilized in 95% ethanol, and optical densities (ODs) were read at 590 nm in a microplate reader (HTS 7000 Plus Bio Assay Reader, Perkin Elmer, Norwalk, Conn.). Experiments were performed in duplicate and repeated twice.

Cellulose and curli production. The production of cellulose and curli was determined as described previously *(23, 27).* The production of curli was characterized using LB (BBL/Becton Dickinson) agar without salt supplemented with 40 mg of Congo red per liter (Sigma, St. Louis, Mo.) and 20 mg of brilliant blue per liter (Sigma). Isolates were streaked onto Congo red plates and incubated for 48 h at 28°C before determining morphotypes by comparing them to control strains. Isolates were grouped into three distinct morphotypes: (i) red, dry, and rough, indicating curli and cellulose production (rdar); (ii) brown, dry, and rough, indicating curli production but a lack of cellulose synthesis (bdar); and (iii) smooth and white, indicating a lack of both curli and cellulose production (saw). Cellulose production was characterized by streaking isolates onto LB plates containing 200 mg of calcofluor (fluorescent brightener 28, Sigma) per liter and incubating at room temperature for 48 h. Cellulose production was judged by comparing the fluorescence of the test strains to that of the control strains under UV light (366 nm). All isolates were screened in duplicate and repeated twice.

Statistical analysis. The absorbance data for each individual observation were scaled against the values obtained for noninoculated control wells of the respective 96-well plates. Resulting negative values were set to zero. For each of the three culture methods, the data from the multiple replicates were pooled, and the mean values were calculated for each isolate. To identify a relationship between the source of the isolate and the degree of crystal violet binding, the isolates were grouped as clinical, meat, and produce. The data for the groups were pooled and analyzed by analysis of variance (ANOVA) ($P < 0.05$; SigmaStat 2.03, SPSS, Inc., Chicago, Ill.) for the effect of (i) isolate source and (ii) culture method. Occurrence of the various morphotypes (or fluorescence on LB plus calcofluor plates) among the three isolate groups was compared using chi-square analysis (SigmaStat). The

TABLE 2. *Biofilm formation by produce-related isolates of* Salmonella

Salmonella serotypes	Food source[b]	Biofilm formation[a]			Morphotype on Congo red[c]	Fluorescence on LB + calcofluor[d]
		LB + 2% glucose	TSB	1/20-TSB		
Anatum F4317	Sprout outbreak	0.105 ± 0.012[a]	0.566 ± 0.067	0.796 ± 0.074	rdar	+
Baildon 61-99	Tomato outbreak	0.018 ± 0.004	0.000 ± 0.000	0.087 ± 0.010	saw	−
Bredeney 3VIPHE	Alfalfa seeds	0.103 ± 0.012	0.573 ± 0.062	0.497 ± 0.046	bdar	−
Entertidis 15159	Orange juice outbreak	0.035 ± 0.005	0.405 ± 0.056	0.449 ± 0.048	saw	−
Gaminara 02-615	Cantaloupe	0.181 ± 0.028	0.327 ± 0.034	0.187 ± 0.019	bdar	−
Gaminara F2712	Orange juice	0.143 ± 0.014	1.242 ± 0.112	0.073 ± 0.012	bdar	−
Hidalgo 02-517-2	Cantaloupe	0.080 ± 0.013	0.126 ± 0.027	0.265 ± 0.027	rdar	+
Infantis F4319	Sprout outbreak	0.027 ± 0.005	0.653 ± 0.065	0.936 ± 0.089	rdar	+
Mbandaka 00-916-1	Cantaloupe	0.056 ± 0.007	0.341 ± 0.035	0.714 ± 0.067	bdar	−
Mbandaka RV1DHE	Alfalfa seeds	0.052 ± 0.006	0.455 ± 0.070	0.382 ± 0.037	bdar	−
Michigan	Cantaloupe outbreak	0.622 ± 0.064	1.259 ± 0.113	0.561 ± 0.056	rdar	+
Montevideo G4639	Tomato outbreak	0.053 ± 0.007	0.820 ± 0.077	0.660 ± 0.075	rdar	+
Muenchen HERV2C	Alfalfa seeds	0.040 ± 0.005	0.102 ± 0.014	0.705 ± 0.067	rdar	+
Newport 02-216	Cantaloupe	0.033 ± 0.007	0.107 ± 0.013	0.494 ± 0.052	rdar	+
Newport H1275	Sprout outbreak	0.485 ± 0.049	1.180 ± 0.107	0.359 ± 0.037	rdar	+
Oranienburg 389	Cantaloupe	0.072 ± 0.009	0.481 ± 0.057	0.629 ± 0.060	rdar	+
Poona 348	Cantaloupe	0.152 ± 0.016	0.802 ± 0.084	0.356 ± 0.038	rdar	+
Poona G-91-1574	Cantaloupe outbreak	0.041 ± 0.009	0.615 ± 0.091	0.128 ± 0.014	saw	−
Poona PTVS1	Cantaloupe outbreak	0.046 ± 0.006	0.203 ± 0.024	0.387 ± 0.041	rdar	+
Poona RM2350	Cantaloupe outbreak	0.1 ± 0.013	0.518 ± 0.081	0.273 ± 0.034	bdar	−
Saint Paul 02-517-1	Cantaloupe	0.075 ± 0.009	0.466 ± 0.056	0.609 ± 0.062	rdar	+
Saphra 97A3312	Cantaloupe outbreak	0.016 ± 0.003	0.270 ± 0.039	0.053 ± 0.008	saw	−
Stanley HO558	Sprout outbreak	0.018 ± 0.004	0.593 ± 0.059	0.366 ± 0.035	saw	−
Typhimurium 45	Cantaloupe	0.169 ± 0.030	0.843 ± 0.086	0.369 ± 0.043	rdar	−
Worthington TX3-1	Alfalfa seeds	0.064 ± 0.007	0.168 ± 0.045	0.969 ± 0.090	rdar	+

[a] Average OD (590 nm) ± standard error from two separate experiments.
[b] All isolates associated with produce outbreaks were clinical isolates.
[c] rdar, red, dry, and rough morphotype indicating curli and cellulose production; saw, smooth and white morphotype indicating lack of curli or cellulose production; bdar, brown, dry, and rough morphotype indicating curli production but lack of cellulose synthesis.
[d] Under long-wave UV (366 nm).

absorbance data were collated on the basis of morphotype and fluorescence and compared using ANOVA.

RESULTS

Results demonstrate that all *Salmonella* isolates produced significant amounts of biofilm when cultivated in the appropriate medium. Mean biofilm formation in each of the three media tested is shown in Tables 1 through 3. Biofilm formation in LB supplemented with 2% glucose was minimal compared to that in TSB or 1/20-TSB (as indicated by the large differences in OD values), indicating that biofilm formation was greatly enhanced in both TSB and 1/20-TSB. Within the isolate groups, only clinical isolates bound significantly more crystal violet in 1/20-TSB than in standard TSB (Table 4). Meat- and produce-related strains had similar ODs, regardless of whether the assay was carried out using TSB or 1/20-TSB.

In assessing biofilm formation by the strains from the three sources, no differences could be detected between isolates when compared using similar media. For example, there was no statistical difference in crystal violet binding between clinical, meat-related, and produce-related strains when all three were tested in 1/20-TSB.

The formation of curli and cellulose was assessed on Congo red plates. Morphotypes were judged by comparing test strains to control strains as follows. *Salmonella* Enteritidis 3934 produces both curli and cellulose and expresses the rdar morphotype. *Salmonella* Enteritidis 942 produces curli but no cellulose and expresses the bdar morphotype. *Salmonella* Enteritidis 1162/97 and 1170/97 produces neither curli nor cellulose and results in the saw morphotype. Morphotypes of the strains tested in this study are presented in Tables 1 through 3. The rdar morphotype was the most prevalent from all three sources, and the distribution of morphotypes was significantly different among the three isolate groups (chi-square $P = 0.004$; Table 5). Interestingly, the saw morphotype was detected only among produce-related isolates. A total of 73, 84, and 56% of clinical, meat-, and produce-related isolates, respectively, produced both curli and cellulose. The bdar morphotype was more prevalent (27 and 24%) in the clinical and produce-related strains than in the meat-related (16%) strains (Table 5).

Cellulose production was further tested by streaking isolates onto LB plates supplemented with calcofluor. Results from these plates were in agreement with results from the Congo red plates, except for produce isolate *Salmonella* Typhimurium 45 (Table 2). All other isolates that were bdar

TABLE 3. *Biofilm formation by meat-related isolates of* Salmonella

Salmonella serotypes	Food source	Biofilm formation[a]			Morphotype on Congo red[b]	Fluorescence on LB + calcofluor[c]
		LB + 2% glucose	TSB	1/20-TSB		
Anatum 313	Ground beef	0.077 ± 0.011[a]	0.612 ± 0.051	0.510 ± 0.066	rdar[b]	+
Derby 5131	Pork	0.070 ± 0.006	0.037 ± 0.009	0.203 ± 0.009	rdar	+
Kentucky 074	Beef	0.059 ± 0.011	0.241 ± 0.044	0.400 ± 0.014	bdar[c]	−
Montevideo 051	Beef	0.404 ± 0.059	0.504 ± 0.051	0.525 ± 0.015	bdar	−
Poona 418	Octopus	0.114 ± 0.013	1.152 ± 0.107	0.223 ± 0.023	rdar	+
Poona 953	Ovine meat	0.061 ± 0.008	0.321 ± 0.054	0.584 ± 0.067	rdar	+
Saint Paul 5130	Pork	0.094 ± 0.006	0.146 ± 0.019	0.362 ± 0.043	rdar	+
Saint Paul FSIS 039	Beef	0.025 ± 0.004	0.392 ± 0.036	0.408 ± 0.053	rdar	+
Typhimurium 026	Beef	0.093 ± 0.009	0.838 ± 0.078	0.893 ± 0.045	rdar	+
Typhimurium 453	Ground beef	0.010 ± 0.002	0.245 ± 0.022	0.293 ± 0.040	rdar	+
Typhimurium Copenhagen 8457	Pork	0.312 ± 0.048	0.510 ± 0.045	0.411 ± 0.020	rdar	+
Typhimurium S-2380	Beef	0.024 ± 0.004	0.173 ± 0.024	0.715 ± 0.073	rdar	+
Hadar 064	Chicken	0.151 ± 0.018	0.490 ± 0.019	0.952 ± 0.032	bdar	−
Hadar MF 61777	Turkey	0.102 ± 0.006	0.858 ± 0.056	0.670 ± 0.020	bdar	−
Heidelberg 258	Ground chicken	0.007 ± 0.002	0.221 ± 0.030	0.293 ± 0.036	rdar	+
Heidelberg 293	Ground turkey	0.021 ± 0.003	0.145 ± 0.023	0.254 ± 0.026	rdar	+
Heidelberg 475	Ground chicken	0.002 ± 0.001	0.674 ± 0.076	0.117 ± 0.018	rdar	+
Kentucky 044	Chicken	0.072 ± 0.013	0.301 ± 0.025	0.572 ± 0.021	bdar	−
Kentucky 479	Ground chicken	0.016 ± 0.003	0.596 ± 0.072	0.241 ± 0.029	rdar	+
Muenster MF 59707	Turkey	0.071 ± 0.030	0.637 ± 0.096	0.307 ± 0.015	rdar	+
Muenster MF 61976	Turkey	0.117 ± 0.002	0.358 ± 0.018	0.438 ± 0.015	rdar	+
Newington 315	Ground chicken	0.027 ± 0.004	0.596 ± 0.065	0.324 ± 0.047	rdar	+
Reading MF 58210	Turkey	0.109 ± 0.003	0.559 ± 0.032	0.523 ± 0.019	rdar	+
Schwarzengrund 214	Ground turkey	0.078 ± 0.008	0.434 ± 0.057	0.475 ± 0.045	rdar	+
Schwarzengrund 351	Ground turkey	0.023 ± 0.003	0.331 ± 0.047	0.277 ± 0.033	rdar	+
Saint Paul 443	Ground turkey	0.028 ± 0.005	0.221 ± 0.023	0.094 ± 0.006	rdar	+
Saint Paul 461	Ground turkey	0.033 ± 0.006	0.292 ± 0.027	0.320 ± 0.041	rdar	+
Thompson 132	Chicken	0.098 ± 0.012	0.237 ± 0.016	0.501 ± 0.018	rdar	+
Thompson 120	Chicken	0.089 ± 0.007	0.676 ± 0.021	0.448 ± 0.015	rdar	+
Typhimurium 209	Ground chicken	0.001 ± 0.001	0.289 ± 0.033	0.549 ± 0.059	rdar	+
Typhimurium 343	Ground chicken	0.001 ± 0.001	0.253 ± 0.032	0.430 ± 0.050	rdar	+

[a] Average OD (590 nm) ± standard error from two separate experiments.
[b] rdar, red, dry, and rough morphotype indicating curli and cellulose production; bdar, brown, dry, and rough morphotype indicating curli production but lack of cellulose synthesis.
[c] Under long-wave UV (366 nm).

TABLE 4. *Summary and comparison of biofilm formation by clinical, produce-related, and meat-related* Salmonella

Isolates	Biofilm formation[a,b]		
	LB + 2% glucose	TSB	1/20-TSB
Clinical	0.064 XA[a]	0.373 YA	0.535 ZA
Meat-related	0.077 YA	0.430 ZA	0.429 ZA
Produce-related	0.111 YA	0.525 ZA	0.452 ZA

[a] OD (590 nm).
[b] Within isolate group, letters (X, Y, Z) indicate significant differences among media ($P < 0.05$, ANOVA); within medium, letter (A) indicates no significant differences among isolate groups ($P > 0.05$, ANOVA).

or saw on Congo red plates were not fluorescent on LB plus calcofluor (Tables 1 through 3).

We attempted to assess whether there was any relationship between morphotype and crystal violet binding. Isolates were sorted by morphotype and then compared by OD values in the various media (Table 6). When grown in LB plus 2% glucose, bdar isolates were significantly higher in crystal violet binding than were rdar and saw isolates. No significant differences were observed between morphotypes when they were grown in TSB. The binding of crystal violet for bdar and rdar strains was statistically higher than that for saw isolates when grown in 1/20-TSB. Note, however, that saw isolates produced the least amount of biofilm in each of the three media.

DISCUSSION

We undertook this study to determine whether produce-related isolates exhibited enhanced biofilm-forming capa-

TABLE 5. *Prevalence of Congo red morphotype and fluorescence on calcofluor plates sorted by isolate origin*[a]

Isolates	Morphotype prevalence			Fluorescence on LB + calcofluor
	rdar	bdar	saw	
Clinical	11/15 (73)	4/15 (27)	0/15	11/15 (73)
Meat-related	26/31 (84)	5/31 (16)	0/31	26/31 (84)
Produce-related	14/25 (56)	6/25 (24)	5/25 (20)	13/25 (52)

[a] Values are number of isolates exhibiting morphotype/total isolates (percentage). Proportions of isolates displaying various morphotypes are significantly different (chi-square $P = 0.004$).

bilities, given our observations of biofilm formation by *Salmonella* on cantaloupe surfaces. Previous authors have demonstrated *Salmonella* biofilm formation on a variety of surfaces, but, to our knowledge, none have investigated whether this was influenced by the source of the test organism. We found isolates from all three sources to be strong biofilm formers. These findings are in agreement with previously published reports on the high capacity of salmonellae to form biofilms on plastic surfaces *(26, 29, 30).* Biofilm formation by produce-related isolates was not significantly higher than that by meat or clinical isolates when tested in LB plus 2% glucose, TSB, or $\frac{1}{20}$-TSB.

Biofilm formation has been shown to be an important determinant in the virulence of a number of pathogenic bacteria. Isolates of *Enterococcus faecalis* associated with endocarditis produced significantly more biofilm than did nonendocarditis isolates *(18).* Djordjevic et al. *(8)* observed that biofilm production of lineage I *Listeria monocytogenes* strains, which contain the majority of strains from clinical cases, was significantly higher than that of lineage II and III strains. *L. monocytogenes* strains that were persistent in food production plants also demonstrated enhanced adherence compared to nonpersistent isolates *(15).* Catheter-related outbreak strains of *Staphylococcus epidermidis* were distinguishable from nonoutbreak strains by their ability to produce biofilms on glass *(9).* All salmonellae tested in this study are able to form strong biofilms, regardless of whether they are clinical isolates, isolates from produce or meat, or related to outbreaks resulting from the consumption of contaminated produce or meat. Interestingly, when these *Salmonella* isolates were regrouped by moving the produce-related and meat-related strains arising from outbreaks to the list of clinical isolates, the ODs of the clinical isolates were significantly higher than those of the remaining meat- and produce-related strains when tested in $\frac{1}{20}$-TSB (data not shown).

We found no differences in biofilm formation between clinical, meat-related, or produce-related isolates in any of our test media. ODs measured for the isolates in LB plus 2% glucose were similar to those reported for *Salmonella* Typhimurium after 18 h of incubation at 30°C *(17).* In addition, ODs measured in TSB were similar to those found by Stepanović et al. *(30)* for moderate-to-strong biofilm-forming strains. The observation that nutrient-limited media ($\frac{1}{20}$-TSB) was effective in promoting biofilm formation agrees with previously published reports *(7, 31).* Transcription of the *csgD* gene, which positively controls curli and cellulose production, has been shown to increase under nutrient-limiting conditions in *Salmonella (13).*

The morphotypes of *Salmonella* Enteritidis isolates from clinical and animal origins have been reported previously *(27).* Solano et al. *(27)* surveyed 63 clinical strains and found 76 and 24% exhibiting the rdar and saw morphotypes, respectively. Animal-related isolates exhibited the rdar morphotype at a rate of 71% *(27).* In contrast, approximately 73% of our clinical isolates and 84% of our meat-related isolates displayed the rdar morphotype. The rdar morphotype has been linked to increased virulence, and it is not surprising that most of the isolates in our collection display rdar *(23).*

Batch culture under static conditions has been shown to be conducive for the production of curli and cellulose *(27).* Both cell surface components have been demonstrated to be important in biofilm formation on abiotic surfaces; therefore, we hypothesized that rdar isolates might exhibit increased crystal violet binding *(5, 27).* When biofilm formation was measured in TSB, the display of the rdar morphotype did not correlate with increased crystal violet binding in the microtiter plate assay compared to the display of the bdar or saw morphotype. Bdar isolates displayed significantly higher crystal violet binding when tested in LB plus 2% glucose. Both rdar and bdar isolates exhibited significantly higher crystal violet binding than did saw strains in $\frac{1}{20}$-TSB. These data indicate that cell surface components other than curli and cellulose may influence biofilm formation and that the cell surface component(s) responsible may vary due to environmental conditions.

Our results indicate that the incidence of cellulose production and curli biosynthesis is different for isolates from

TABLE 6. *Relationship between isolate morphotypes and biofilm formation as assessed by the crystal violet binding assay*

Morphotype	Relationship between morphotype and OD		
	LB + 2% glucose	TSB	$\frac{1}{20}$-TSB
bdar	0.119 A[a]	0.499 A	0.452 A
rdar	0.083 B	0.445 A	0.486 A
saw	0.026 B	0.377 A	0.217 B

[a] Average OD (590 nm) of isolates of a given morphotype in the specified medium. Within the medium, letters (A, B) indicate no significant differences among isolate groups ($P < 0.05$, ANOVA).

a variety of sources. Cellulose-deficient strains were found only among produce isolates. Barak et al. *(2)* found that *Salmonella* adhered more strongly to alfalfa sprouts than did *Escherichia coli* O157:H7 and postulated that this resulted from the lack of curli production by *E. coli* O157:H7. Eighty percent of the produce-related isolates produced curli. All of the produce-related strains that expressed the saw morphotype were from produce-related outbreaks and not from collected food samples. This may further underscore the importance of curli in attachment to produce surfaces.

The production of both cellulose and curli is important for the survival and persistence of *Salmonella* on surface environments *(22, 23)*. Biofilm formation by human pathogens on fresh produce is not well characterized. While it is clear that *Salmonella* initiates biofilm formation after contacting produce surfaces *(1, 4)*, the role of these biofilms in protecting the pathogen against aqueous sanitizers is not clear. The reduced efficacy of sanitizers on attached bacteria lends credence to the hypothesis that bacteria embedded within biofilms are more resistant (especially after 1 or more days of storage) to inactivation. Our results demonstrate that biofilm formation is not influenced by the origin of the test isolate. The role of biofilm formation, curli, and cellulose in establishing bacteria on the surface of fruits and vegetables must be characterized in order to put forth more effective postharvest intervention treatments.

ACKNOWLEDGMENTS

The authors thank Carlos Gamazo of the University of Navarra (Spain) for providing control strains, William Fett, Pina Fratamico, and Vijay Juneja for access to their culture collections, and Peter Irwin for use of the microplate reader. The authors also thank Gabe Hoffman and Danielle Signorelli for technical assistance. This study was supported in part by the U.S. Department of Agriculture Integrated Research, Education, and Extension grant no. 2002-51110-01987 through a subcontract from Trevor Suslow, Department of Vegetable Crops, University of California, Davis.

REFERENCES

1. Annous, B. A., A. Burke, and J. E. Sites. 2004. Surface pasteurization of whole fresh cantaloupes inoculated with *Salmonella* Poona or *Escherichia coli*. *J. Food Prot.* 67:1876–1885.
2. Barak, J. D., L. C. Whitehand, and A. O. Charkowski. 2002. Differences in attachment of *Salmonella enterica* serovars and *Escherichia coli* O157:H7 to alfalfa sprouts. *Appl. Environ. Microbiol.* 68: 4758–4763.
3. Beuchat, L. R. 2002. Ecological factors influencing survival and growth of human pathogens on raw fruits and vegetables. *Microb. Infect.* 4:413–423.
4. Brandl, M. T., and R. E. Mandrell. 2002. Fitness of *Salmonella enterica* serovar Thompson in the cilantro phyllosphere. *Appl. Environ. Microbiol.* 68:3614–3621.
5. Cookson, A. L., W. A. Cooley, and M. J. Woodward. 2002. The role of type 1 and curli fimbriae of Shiga toxin–producing *Escherichia coli* in adherence to abiotic surfaces. *Int. J. Med. Microbiol.* 292: 195–205.
6. Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: a common cause of persistent infections. *Science* 284: 1318–1322.
7. Dewanti, R., and A. C. Wong. 1995. Influence of culture conditions on biofilm formation by *Escherichia coli* O157:H7. *Int. J. Food Microbiol.* 26:147–164.
8. Djordjevic, D., M. Wiedmann, and L. A. McLandsborough. 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Appl. Environ. Microbiol.* 68:2950–2958.
9. Dunne, W. M. 2002. Bacterial adhesion: seen any good biofilms lately? *Clin. Microbiol. Rev.* 15:155–166.
10. Fett, W. F. 2000. Naturally occurring biofilms on alfalfa and other types of sprouts. *J. Food Prot.* 63:625–632.
11. Fett, W. F., and P. H. Cooke. 2003. Scanning electron microscopy of native biofilms on mung bean sprouts. *Can. J. Microbiol.* 49:45–50.
12. Genevaux, P., S. Muller, and P. Bauda. 1996. A rapid screening procedure to identify mini-Tn10 insertion mutants of *Escherichia coli* K-12 with altered adhesion properties. *FEMS Microbiol. Lett.* 142: 27–30.
13. Gerstel, U., and U. Romling. 2003. The *csgD* promoter, a control unit for biofilm formation in *Salmonella typhimurium*. *Res. Microbiol.* 154:659–667.
14. Joseph, B., S. K. Otta, I. Karunasagar, and I. Karunasagar. 2001. Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. *Int. J. Food. Microbiol.* 64:367–372.
15. Lunden, J. M., M. K. Miettinen, T. J. Autio, and H. J. Korkeala. 2000. Persistent *Listeria monocytogenes* strains show enhanced adherence to food contact surface after short contact times. *J. Food Prot.* 63:1204–1207.
16. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607–625.
17. Mireles, J. R., II, A. Toguchi, and R. M. Harshey. 2001. *Salmonella enterica* serovar Typhimurium swarming mutants with altered biofilm-forming abilities: surfactin inhibits biofilm formation. *J. Bacteriol.* 183:5848–5854.
18. Mohamed, J. A., W. Huang, S. R. Nallapareddy, F. Teng, and B. E. Murray. 2004. Influence of origin of isolates, especially endocarditis isolates, and various genes on biofilm formation by *Enterococcus faecalis*. *Infect. Immun.* 72:3658–3663.
19. Monier, J. M., and S. E. Lindow. 2003. Differential survival of solitary and aggregated bacterial cells promotes aggregate formation on leaf surfaces. *Proc. Natl. Acad. Sci. USA* 100:15977–15982.
20. Morris, C. E., J.-M. Monier, and M.-A. Jacques. 1997. Methods for observing microbial biofilms directly on leaf surfaces and recovering them for isolation of culturable microorganisms. *Appl. Environ. Microbiol.* 63:1570–1576.
21. Morris, C. E., J.-M. Monier, and M.-A. Jacques. 1998. A technique to quantify the population size and composition of the biofilm component in communities of bacteria in the phyllosphere. *Appl. Environ. Microbiol.* 64:4789–4795.
22. Römling, U. 2002. Molecular biology of cellulose production in bacteria. *Res. Microbiol.* 153:205–212.
23. Römling, U., W. Bokranz, W. Rabsch, X. Zogaj, M. Nimtz, and H. Tschäpe. 2003. Occurrence and regulation of the multicellular morphotype in *Salmonella* serovars important in human disease. *Int. J. Med. Microbiol.* 293:273–285.
24. Römling, U., M. Rohde, A. Olsen, S. Normark, and J. Reinkoster. 2000. AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella typhimurium* regulates at least two independent pathways. *Mol. Microbiol.* 36:10–23.
25. Römling, U., W. D. Sierralta, K. Eriksson, and S. Normark. 1998. Multicellular and aggregative behaviour of *Salmonella typhimurium* strains is controlled by mutations in the *agfD* promoter. *Mol. Microbiol.* 28:249–264.
26. Sinde, E., and J. Carballo. 2000. Attachment of *Salmonella* spp. and *Listeria monocytogenes* to stainless steel, rubber and polytetrafluoroethylene: the influence of free energy and the effect of commercial sanitizers. *Food Microbiol.* 17:439–447.
27. Solano, C., B. Garcia, J. Valle, C. Berasain, J.-M. Ghigo, C. Gamazo, and I. Lasa. 2002. Genetic analysis of *Salmonella enteritidis* biofilm formation: critical role of cellulose. *Mol. Microbiol.* 43:793–808.
28. Solano, C., B. Sesma, M. Alvarez, T. J. Humphrey, C. J. Thorns, and C. Gamazo. 1998. Discrimination of strains of *Salmonella enteritidis* with differing levels of virulence by an in vitro glass adherence test. *J. Clin. Microbiol.* 36:674–678.

29. Sommers, E. B., J. L. Schoeni, and A. C. L. Wong. 1994. Effect of trisodium phosphate on biofilm and planktonic cells of *Campylobacter jejuni*, *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella typhimurium*. *Int. J. Food Microbiol.* 22:269–276.
30. Stepanović, S., I. Ćirković, V. Mijač, and M. Švabić-Vlahović. 2003. Influence of incubation temperature, atmosphere and dynamic conditions on biofilm formation by *Salmonella* spp. *Food Microbiol.* 20: 339–343.
31. Stepanović, S., I. Ćirković, L. Ranin, and M. Švabić-Vlahović. 2004. Biofilm formation by *Salmonella* spp. and *Listeria monocytogenes* on plastic surface. *Lett. Appl. Microbiol.* 38:428–432.
32. Ukuku, D. O., and W. F. Fett. 2002. Relationship of cell surface charge and hydrophobicity to strength of attachment of bacteria to cantaloupe rind. *J. Food Prot.* 65:1093–1099.
33. Ukuku, D. O., and G. M. Sapers. 2001. Effect of sanitizer treatments on *Salmonella* Stanley attached to the surface of cantaloupe and cell transfer to fresh-cut tissues during cutting practices. *J. Food Prot.* 64:1286–1291.
34. Zogaj, X., M. Nimtz, M. Rohde, W. Bokranz, and U. Römling. 2001. The multicellular morphotypes of *Salmonella typhimurium* and *Escherichia coli* produce cellulose as the second component of the extracellular matrix. *Mol. Microbiol.* 39:1452–1463.